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CYTOLOGICAL ABNORMALITIES PRODUCED BY EXPERIMENTAL TEMPERATURE SHOCK ON TRITURUS TOROSUS EMBRYOS[1]

CATHERINE HENLEY
University of North Carolina

Introduction

The importance of a complete chromosome complement in normal development is obvious when one considers the evidence that the chromosomes are the carriers of genes governing the characters of the organism. It is of considerable theoretical and practical interest to ascertain the effects of addition or loss of single chromosomes, or of chromosome sets.

Most of the early work in the field of developmental genetics was done with plants, until publication of the reports of Fankhauser (1938 and subsequent papers). These reports indicated the value of a tail-tip method for the cytological study of amphibian larvae. The technique utilized is sufficiently simple that systematic studies can be carried out on a large scale, to determine the incidence of heteroploidy in a natural population of salamander larvae. It was with the view of making such a study on *Triturus torosus* that investigations were begun in 1947 on larvae developing from eggs which had been shipped to Chapel Hill from California, in a mixture of ice and water. These larvae showed a very high incidence of mosaic heteroploidy (43 per cent in a sample of 126 larvae), and of severe mitotic abnormalities of various types. The results of this study will be presented in a separate paper.

The possibility existed that such a frequent occurrence of mosaicism was present in a natural population, as well as in the shipped material. Cytological study of a large group of *T. torosus* larvae collected in nature as young embryos and raised in the laboratory indicated that this was not so (Costello and Henley, 1948, 1949). It was further demonstrated that larvae raised from eggs collected in very cold water had a higher incidence of mitotic abnormality than those collected from warmer water. This finding, coupled with similar work reported by Böök (1943, 1945) and Barber and Callan (1943) implied that low temperature might be at least one of the factors responsible for the effects observed in the shipped embryos. Experiments were therefore planned to test the effects of cold treatment applied at

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various stages of development, from a period shortly after insemination through the tail-bud stage, in *T. torosus*.

Some of the data included here were communicated earlier in a preliminary note (Henley, 1948).

The study was carried on under the direction of Dr. D. P. Costello, to whom the author is very grateful for his stimulating interest and his helpful criticisms. The experiments were performed at the School of Biological Sciences of Stanford University during the winter and spring of 1948; it is a pleasure to acknowledge the many courtesies extended during that time by Dr. Douglas M. Whitaker, and Dr. Victor C. Twitty and his co-workers. The accompanying photomicrographs were made by Dr. Costello.

Methods

The embryos used in these experiments were raised in the laboratory at Stanford University from *Triturus torosus* eggs collected at Los Trancos and Ukiah, California, and from eggs laid in the laboratory by female salamanders collected at Ukiah, Portola and Mt. Hamilton. In all cases, the embryos treated in 1948 were raised within a constant temperature range (13 – 15° C.) except during the experimental treatment. Usually, several egg clutches were treated simultaneously, in a large fingerbowl. Shortly before the time of hatching, the egg masses were segregated, so that embryos from a given clutch could be identified. In the 1948 experiments, non-chlorinated Searsville Lake water was used and the larvae were isolated in separate paraffined paper ice-cream cups according to the method described by Costello and Henley (1949). All animals were photographed at the time of the first clipping (Fig. 1). MS-222 (1 : 2000 in distilled water) was used for anesthetizing the larvae for photography and tail-clipping.

All the 1948 cold treatments reported here involved a temperature shock. Embryos at various stages of development from before cleavage through tail-bud stages were placed in fingerbowls of pre-chilled water maintained at the desired temperature. At the end of the treatment, they were transferred back to the original fingerbowl of water at room temperature and allowed to continue development. The effects of temperatures from 0° (mixture of ice and water) to 8° C. were tested for varying periods of time, as indicated in Table 1. In all cases, the embryos were left within the jelly mass until they hatched normally.

Appropriate controls were kept for the various series; details from the results of a study of these untreated larvae are discussed by Costello and Henley (1949).

When the larvae had hatched and feeding was about to begin, they were photographed and assigned serial numbers. The distal one-third of the tail-tip was clipped, fixed and stained as described by Costello and Henley (1949). After an interval of approximately 12 days, the regenerated tip was likewise amputated and prepared for cytological study. These "second" tips are far superior to "first" tips, since yolk resorption is completed and more mitoses are usually present. All tips were stained with Harris's acid haematoxylin.

Proper feeding is essential if good cytological preparations are to be obtained; an adequate supply of Artemia nauplii was kept in the cups with the larvae to serve as food. These nauplii were cultured in a strong brine solution and washed into fresh lake water before being fed to the salamander larvae.

I am indebted to Dr. Costello for the use of 27 tail-tip preparations of *T. torosus* larvae, made by him in 1942. These tips were derived from embryos which had been cold-treated 5 to 80 minutes after insemination, for periods of 18 to 23 hours. No preparations from newly regenerated tail-tips of this group were prepared and only first clippings are included in the data. The larvae were raised at laboratory room temperature. The cold treatments did not involve temperature shock, the eggs having been chilled gradually and allowed to return slowly to room temperature.

The tail-tip epithelium was studied at a magnification of 210 ×, and any figures of special interest noted on a small "map" of the tip. Using an oil immersion ob-

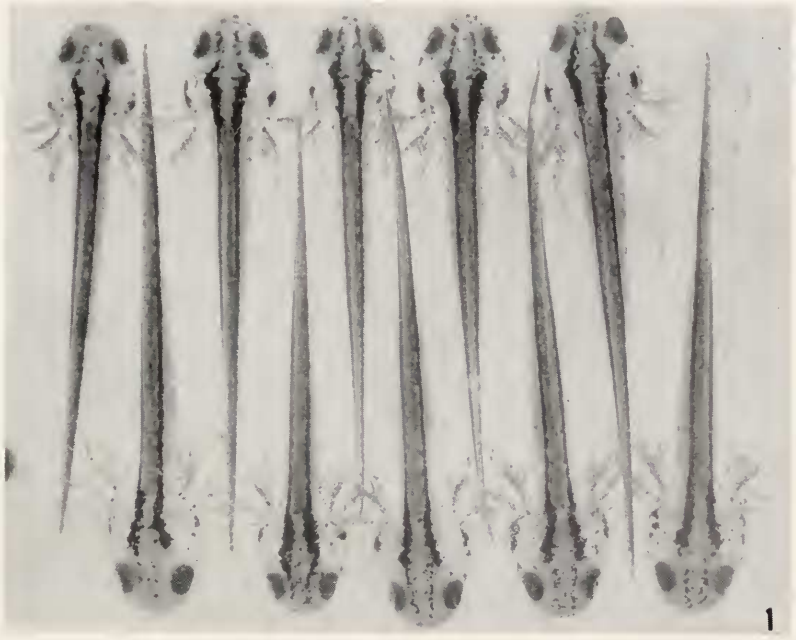

FIGURE 1. A group of *T. torosus* larvae, cold-treated at the gastrula stage for 2 hours at 0° C.; photographed just before their tail-tips were clipped the first time. All ten individuals are normal in general morphology and pigment pattern, but cytologically abnormal to varying degrees. Magnification: 4.5 ×.

jective and 10 × compensating oculars, camera lucida drawings of nuclear size were then made of an area on the dorsal side of each tip, as described by Costello and Henley (1949). Mitotic figures were drawn with the camera lucida at a magnification of 2100 ×, in order to establish chromosome counts.

Three criteria were utilized in classifying the tail-tips: variations in nuclear size or nucleolar number, the presence of abnormal mitotic configurations, and the presence of heteroploid metaphases. The relative value of these criteria has been discussed in the paper of Costello and Henley (1949); they conclude that only decisive chromosome counts of clear mitotic figures are reliable for a definite diagnosis of chromosomal mosaicism, but point out that nucleolar number and nuclear size

TABLE I

The incidence of cytological abnormalities in tail-tip preparations of T. torosus larvae subjected to cold at various stages of development

Stage treated	Treatment	First tips					Second Tips				
		No. tips	Diploid	Variable nuclei	Abnor. mitoses	Mosaics	No. tips	Diploid	Variable nuclei	Abnor. mitoses	Mosaics
10 min. a.i.*	0°–45 mins.	12	4 33%	5 42%	3 25%	0 0	7	0 0	5 71%	2 29%	0 0
Cleavage, blastula	0°–4 hrs.	115	51 44%	49 43%	14 12%	1 0.9%	82	15 18%	28 34%	34 41%	5 7%
Blastula	0°–1 hr.	28	4 14%	11 39%	12 43%	1 4%	20	3 15%	7 35%	6 30%	4 20%
Blastula	8°–3 days;										
	5°–12 days	10	3 30%	7 70%	0 0	0 0	4	1 25%	0 0	3 75%	0 0
Gastrula	0°–2 hrs.	8	4 50%	2 25%	2 25%	0 0	1	0 0	1 100%	0 0	0 0
Gastrula	0°–2 hrs.	35	10 29%	11 31%	14 40%	0 0	33	5 15%	2 6%	24 73%	2 6%
Neurula	0°–3 hrs.	30	12 40%	13 43%	4 13%	1 3%	3	2 67%	0 0	1 33%	0 0
Neurula	4°–2 hrs.	4	1 25%	3 75%	0 0	0 0	3	0 0	2 67%	1 33%	0 0
Tail-bud	0°–4 hrs.	150	64 43%	55 37%	30 20%	1 0.6%	141	45 32%	62 44%	31 22%	3 2%
Totals		392	153	156	79	4	294	71	107	102	14
Percentages			39%	40%	20%	1%		24%	36%	35%	5%
Controls		582	423	105	53	1	429	284	88	55	2
Percentages			73%	18%	9%	0.17%		66%	20%	13%	0.5%
1942 Material:											
A. 5 min. a.i.*	4°–23 hrs.	5	0	5	0	0					
B. 20 min. a.i.*	0°–1½ hrs.;										
	4°–19 hrs.	6	1	1	4	0					
C. 30 min. a.i.*	4°–19 hrs.	3	2	1	0	0					
D. 80 min. a.i.*	4°–18 hrs.	13	2	4	2	5					
Totals		27	5	11	6	5					

* "a.i." After insemination.

are often of considerable value as adjuncts to chromosome counts in classifying a given tail-tip. Unfortunately, reliable chromosome counts are often very difficult to make, especially in the experimentally cold-treated material reported here. Early observations on shipped embryos (Costello and Henley, 1947) have indicated that the occurrence of mitoses having a deficiency or a superfluity of single chromosomes may be quite high. Such figures are often crowded, which makes the detection of aneuploid mitoses difficult.

Sometimes tail-tips had more than one type of abnormality; in these cases, classification was based on the most definitely radical of the atypical features. For example, a tip having both marked variation in nuclear size and nucleolar number, and anaphases with lagging elements was classed with the larvae showing abnormalities of mitosis. Similarly, a tip with variable nuclear size, abnormal mitoses and some metaphases having demonstrably more or less than the diploid number of 22 chromosomes (Henley and Costello, 1947) was classified among the mosaic individuals.

Results

The results of a study of 392 "first" tips and 294 regenerated tips are summarized in Table 1, together with the findings obtained from cytological examination of the control tips. No polyploid individuals were observed in either the control or the experimental groups. Among the "first" tips, 153 (39 per cent) were normal diploids, 156 (40 per cent) had nuclei of variable size or abnormalities of nucleolar number, 79 (20 per cent) had abnormal mitoses, and 4 (1 per cent) were chromosomal mosaics. All four of these mosaics appeared to be diploid triploid individuals, although confirmation by chromosome counts was possible in only one case. In the remaining three, it was obvious that more than 22 chromosomes were present in some of the metaphases. Of the 294 regenerated tips, 71 (24 per cent) were clearly diploid, 107 (36 per cent) had nuclei of variable size or variations in nucleolar number, 102 (35 per cent) had abnormal mitoses, and 14 (5 per cent) were mosaics. Included among the mosaics were four diploid hyperdiploid individuals, seven diploid/triploids, one diploid/triploid/tetraploid, one possible diploid/tetraploid, and one hypodiploid/diploid hyperdiploid/triploid. Five of these diagnoses could be confirmed by chromosome counts, and photographic records were made of two more.

Among the "first" tips, the incidence of all types of abnormality was generally lower than among the "second" tips; this was also found to be true of the control animals. The incidence of abnormality in the experimental group was, in almost every case, double or more than double that found among the controls.

The effectiveness of the various treatments in producing mosaicism and other abnormalities is shown in Table 2, where the data are grouped according to the stage treated, without respect to the temperature or duration of treatment. It appears that the early stages of development, through gastrulation, are somewhat more sensitive to the effects of temperature shock than neurulae and tail-buds. This is especially evident in the regenerated "second" tips.

"First" tips from 27 larvae experimentally subjected in 1942 to low temperature were also studied. These had all been cold-treated within 80 minutes after insemination for relatively long periods of time (18–23 hours). Five of the larvae (19

per cent) were normal diploids, 11 (41 per cent) had nuclei of variable size or abnormalities in nucleolar number, 6 (22 per cent) had abnormal mitoses, and 5 (19 per cent) were chromosomal mosaics. Included among these mosaics were three diploid/triploids, one diploid/triploid/tetraploid, and one diploid/triploid/pentaploid. All except a single diploid/triploid were confirmed by direct chromosome counts.

The variation in nuclear size and nucleolar number noted in these experimentally cold-treated larvae was considerably more pronounced, both qualitatively and quantitatively, than in the control tips. It is of interest to note that there was no direct correlation between nuclear size and nucleolar number. Many of the nuclei within the normal range of size had three or even more nucleoli. Conversely, very large nuclei often had only two nucleoli.

In many of the heteroploid and a few of the diploid metaphases the chromosomes were apparently split (Fig. 6). When counts were made of such figures, each pair of chromatids was counted as a unit.

TABLE II

The percentage of cytological abnormalities found in tail-tip preparations of T. torosus larvae subjected to cold at various stages of development

Stage treated	First tips				Second tips			
	2 N	Var. nuc.	Abn. mit.	Mos.	2 N	Var. nuc.	Abn. mit.	Mos.
10 mins. a.i.*	33%	42%	25%	0	0	71%	29%	0
Blastula	38%	44%	17%	1%	18%	33%	40%	8%
Gastrula	33%	30%	37%	0	14%	9%	70%	6%
Neurula	38%	47%	12%	3%	33%	33%	33%	0
Tail-bud	43%	37%	20%	0.06%	32%	44%	22%	2%
1942 Material:								
5 to 80 mins. a.i.*	19%	41%	22%	18%	—	—	—	—

* After insemination.

Cell condensations which appear identical with the lateral line organs were in an atypical position in many of the preparations. Usually, no visible deficiency was present in the normal arrangement, so that the displaced condensations may represent supernumerary lateral line organs.

Blister-like protuberances were occasionally observed on interphase nuclei; in some instances, blebs of chromatin material appeared to have been extruded from nearby nuclei. These blebs retain the organized appearance of normal complete nuclei, and are thus distinct from the nuclear débris described by Costello and Henley (1949). Abnormal nuclei, similar to those observed in control larvae collected from very cold water, were present in some of the experimentally cold-treated tail-tips. Such nuclei are characterized by the presence of a highly basophilic periphery, surrounding a relatively homogeneous central region which stains less intensely.

Among the more striking mitotic abnormalities observed were masses of relatively uncondensed chromatin, sometimes occurring as isolated configurations, and

occasionaly in conjunction with metaphase mitoses. In a few cases, these large masses of basophilic material appeared to be in prophase. An example of this type is shown in Figure 2; here the chromatin mass is connected by a very thin bridge to an adjoining atypical prophase figure, and has many lobes. Three dark bodies (not visible in the photomicrograph) are present in the main mass of chromatin. It is possible that these represent nucleoli, although they do not have the usual appearance of such structures. Normally, nucleoli are not visible in prophase configurations in these tail-tips. In some instances, a chromatin mass in association with a metaphase figure appeared to represent uncondensed chromosomes of the figure, since only a very few normal chromosomes were present.

Metaphase plates occurred in which one or more chromosomes were abnormally oriented on the spindle. Sometimes this abnormality of orientation was so pronounced as to indicate that the chromosome or chromosomes had completely lost spindle connections and were loose in the cytoplasm. Fusion of metaphase chromosomes was apparent in several tail-tips; various steps in the process of fusion were noted, from a stage at which the identity of most of the components was still established to one where only a mass of chromatin was visible. Such chromatin masses are distinguished from the type shown in Figure 2 by the fact that they retain the characteristic ring-shape of a normal metaphase in polar view. Furthermore, the chromatin is much less diffuse than in the unorganized masses of basophilic material. Similar fused configurations were observed in what appear to be telophase (Fig. 9) and anaphase mitoses.

By far the largest number of mitotic abnormalities was observed in anaphases and telophases, confirming an observation made on untreated control material. One of the common types of aberration is shown in Figure 10, where connections persist between several chromosomes of the separating elements. A less radical "sticky" configuration is shown in Figure 11, where only a single connection is present. In the anaphase shown in Figure 12, one of the chromosomes or chromosome fragments appears to be lagging and atypical in its orientation on the spindle. Other examples of this type of abnormality have also been noted. In one case, an apparently normal chromosome is lying at right angles across the spindle; it appears to have lost its connection entirely. Similar examples of "lost" and atypically oriented chromosomes or chromosome fragments are shown in Figures 13, 14, and 18. The fragments may be incorporated into one of the daughter cells as in Figure 13, or lie on the spindle between the two poles as in Figure 14. In the former case, they might contribute to the formation of an aneuploid cell if complete chromosomes are involved. However, in cases where the chromosome material is between the two poles, it seems likely that eventually it would be expelled into the cytoplasm. An abnormal orientation of anaphase poles is shown in Figure 15; one group of daughter chromosomes is lying with the center of orientation almost at right angles to that of the other group. The spindle area of this anaphase shows clearly the characteristic staining properties noted at this stage. Ordinarily, during all stages of mitosis except anaphase and early telophase, only the chromosomes and chromatin material stain blue, leaving the background completely colorless. However, at anaphase, the region between the groups of daughter chromosomes often appears clearly defined as a light blue area. Recognizable spindle fibres have not been definitely identified in the present material using

this staining technique, although they seem to be discernible in some anaphase configurations.

The connections between separating anaphase and telophase groups may persist, so that in some preparations, a high percentage of interphase nuclei with connecting "bridges" is present. Occasionally this connection is thin and attenuated; in other instances it is a rather substantial structure. All gradations between the two extremes were noted, as well as cases where the thin connection appears to have been broken, leaving only a slender projection from each daughter nucleus. Sometimes persistent connections have very sharp bends at the middle, as though some localized pressure were being exerted at that point. In many cases of connected daughter nuclei, the components are asymmetrical, so that one pole is normal in size or larger than normal, while the other is considerably smaller.

In some tail-tips, anaphases or telophases were observed in which nuclear débris, of the type described by Costello and Henley (1949) is present on the spindle or incorporated into one of the daughter nuclei. The characteristic globules of the débris are usually surrounded by a clear halo, which appears well defined against the light blue background of the spindle area.

One remarkably clear tripolar anaphase was found in a tail-tip from a larva cold-treated at the blastula stage (Fig. 16). In this figure, two of the poles appear considerably smaller than the third. The chromosomes in all three groups seem to

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PLATE I

Figures 2–18 are photomicrographs of chromatin configurations in the tail-tip epithelium of *T. torosus* larvae; from whole-mount preparations stained with Harris's acid haematoxylin. Magnification: 1090 ×.

FIGURE 2. A large mass of chromatin which appears to be in prophase. Two lobes are shown connected to the main mass, at the bottom of the photomicrograph. The connection of the lobe at the left is rather thick, while that of the lobe at the right is quiet slender. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 3. Normal diploid metaphase in polar view, with 22 chromosomes readily countable. Compare with Figures 4–9.

FIGURE 4. Triploid (or possibly tetraploid) metaphase. The figure is too crowded for an accurate count, but it is obvious that far more than the normal diploid number of chromosomes is present. From a larva treated at the gastrula stage for 2 hours at 0° C.

FIGURE 5. Triploid metaphase. Twenty-eight chromosomes could be counted accurately and more were present. From a larva treated at the blastula stage for 4 hours at 0° C.

FIGURE 6. Probable tetraploid metaphase. Note the split condition of the chromosomes and the departure from the normal ring configuration of a metaphase in polar view. From a larva treated 80 minutes after insemination at 4° C. for 18 hours (1942 material).

FIGURE 7. Pentaploid metaphase. One sector of the mitosis appears to be on a different level than the remainder, and is therefore out of focus in the photomicrograph. From a larva treated at the blastula stage for 1 hour at 0° C.

FIGURE 8. Hypodiploid metaphase. Thirteen chromosomes were clearly countable, and one or two others could not be traced accurately enough to be included in the count. An adjacent metaphase (not shown in the photomicrograph) is clearly hyperdiploid, with more than 22 chromosomes present. The two figures are apparently in separate cells, although the cell boundaries are not visible. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 9. "Arrested" configuration. It is not clear whether this is an atypical telophase or a compound metaphase. Two centers of orientation are present; the chromosomes are beginning to lose their identity and to fuse with one another. From a larva treated at the gastrula stage for 2 hours at 0° C.


2


3


4


5


6


7


8


9

be normal. A possible tetrapolar figure is shown in Figure 17, although the abnormal condensation of chromosomes in two of the elements makes it difficult to interpret the relationship of the four poles to one another. The orientation of all four groups toward a common center suggests that this may represent a tetrapolar anaphase. Superficially, the configuration shown in Figure 18 also resembles a tetrapolar mitosis. However, the morphology of the chromosomes in the lower pair of poles indicates that this group of chromosomes is in a different stage of anaphase than the other two.

In general, there seemed to be no correlation between the severity of the cold treatment and the nature of the mitotic abnormalities produced. The greatest number of mosaics (as shown in Table 1) was obtained by treating embryos shortly after insemination, and at the blastula and gastrula stages.

The appearance of the cold-treated larvae is often remarkably normal (Fig. 1), even though the tail-tip preparations from such animals may have quite radical anomalies of mitosis and of chromosome number. This is in accord with unpublished observations made on shipped *T. torosus* and experimentally cold-treated

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PLATE II

FIGURE 10. "Sticky" anaphase. The groups of daughter chromosomes have begun to separate, but connections persist in at least three regions. From a larva treated at the gastrula stage for 2 hours at 0° C.

FIGURE 11. "Sticky" anaphase. In this configuration only one connection persists between the daughter elements, which have completed or almost completed their movement. The dark granules are embryonic pigment. The outline of the spindle area is faintly discernible in some regions. From the same larva as the metaphase shown is Figure 7, treated at the blastula stage for 1 hour at 0° C.

FIGURE 12. Anaphase with disoriented chromosome or chromosome fragment. The "lost" element appears to be devoid of a spindle attachment; its apparent adherence to the end of one of the daughter chromosomes is unusual in this material. Granules of embryonic pigment are visible in the spindle area, which is stained the light blue characteristic at this stage. From a larva treated at the blastula stage for 4 hours at 0° C.

FIGURE 13. Anaphase or early telophase with chromosome or chromosome fragment "lost" at one pole. The figure is surrounded by a vacuolated area which appears light. Cytokinesis has begun and part of the new cell boundary is visible. From the same larva as Figure 9, treated at the gastrula stage for 2 hours at 0° C.

FIGURE 14. Telophase with two chromosomes or chromosome fragments lagging on the spindle. These appear to be homologous daughter chromosome fragments which have been lost from their respective polar groups. From a larva treated at the blastula stage for 1 hour at 0° C.

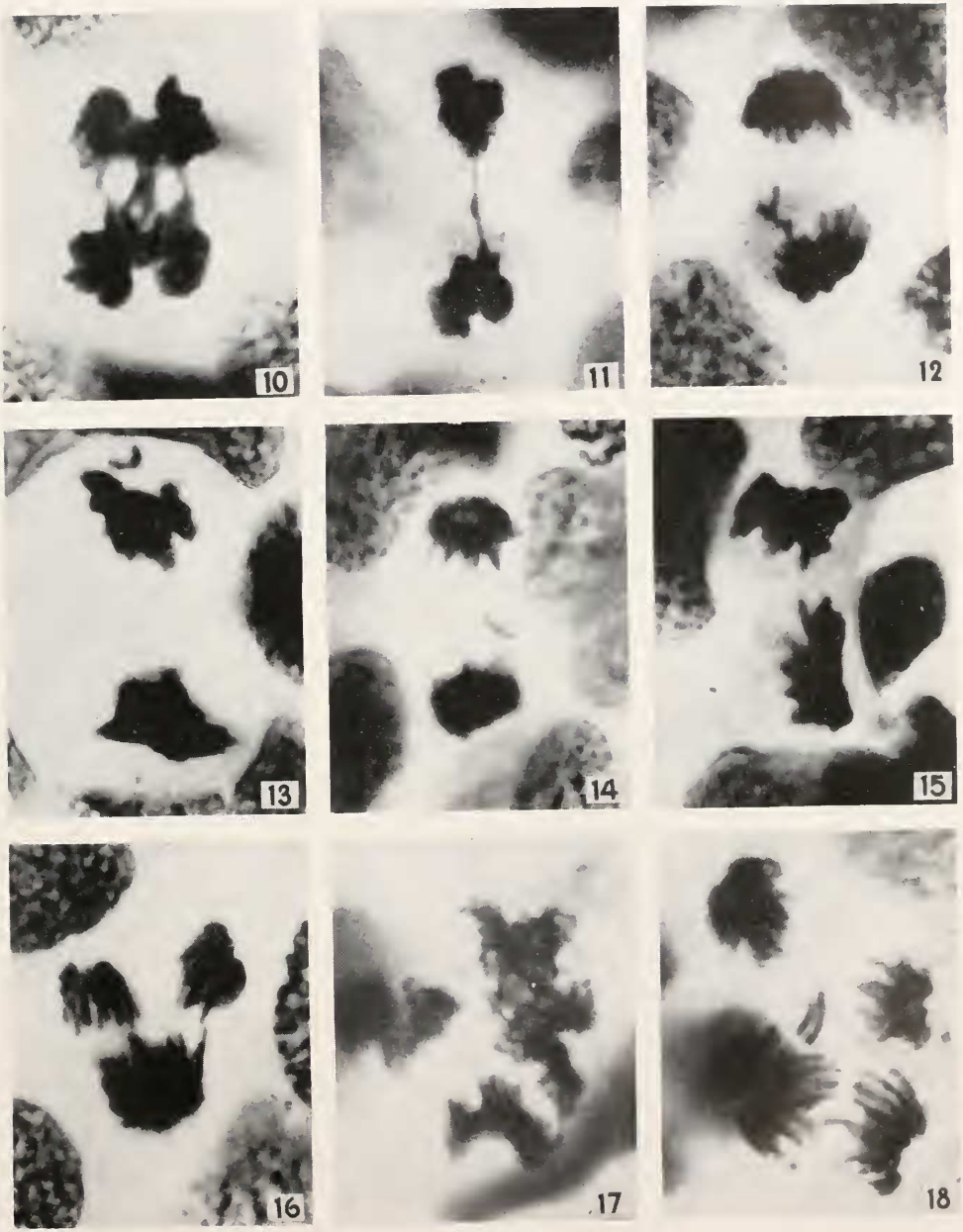
FIGURE 15. Anaphase with bent spindle. The center of orientation of one of the poles is almost at right angles to that of the other. The lightly-stained spindle area characteristic of this stage is unusually clear in this photomicrograph. From a larva treated at the tail-bud stage for 4 hours at 0° C.

FIGURE 16. Tripolar anaphase. This is an exceptionally clear example of a multipolar figure. Two of the poles appear to have fewer chromosomes than the third, but are too crowded to count. The edge of a normal prophase is visible at the right of the photograph. From the same larva as figure 12 treated at the blastula stage for 4 hours at 0° C.

FIGURE 17. Tetrapolar anaphase. The arrangement of the chromosomes in two of the four groups is irregular. All four poles appear to be oriented toward the center. From a larva treated at the blastula stage for 1 hour at 0° C.

FIGURE 18. Two adjacent anaphases with a pair of homologous daughter chromosomes lost from one of the spindles. The chromosome movements have progressed to different degrees in the two figures. From a larva treated at the tail-bud stage for 4 hours at 0° C.

PLATE II



Amblystoma punctatum larvae. In some cases, however, the atypical chromosome condition is reflected in the general morphology. Larvae have also been studied in which all the evidence indicates a normal diploid chromosome condition, even though the animal was abnormal in appearance.

Discussion

A. The absence of polyploidy

Fankhauser and Griffiths (1939) devised an effective and simple method for the production of triploids in *Triturus viridescens*, based on the earlier experiments of Rostand (1934). They found that refrigeration of the eggs shortly after insemination resulted in a high percentage of triploid individuals. This effect was attributed to a suppression of the second maturation spindle which normally gives off the second polar body shortly after fertilization (Fankhauser and Griffiths, 1939). Recently, Fankhauser and Godwin (1948) have demonstrated that the most important effect of heat treatment in producing triploid *Triturus viridescens* eggs is a submergence of the mitotic figure below the egg cortex, giving two haploid egg pronuclei. Fankhauser and Watson (1942) and Briggs (1947) also have found that heat treatment shortly after insemination is effective in producing triploids. It appears that temperature shock is the important factor in all these cases, since abrupt removal of the eggs to and from the experimental temperature bath produces a considerably higher incidence of triploidy than less drastic transfers.

Possible explanations for the absence of polyploidy in a "natural population" of untreated *T. torosus* larvae have been discussed by Costello and Henley (1949). It is suggested that balanced lethal factors are often present in the diploids of this species. Triploids, having an extra dose of one or more of the lethal genes, might die early in development, due to a lethal imbalance. Presumably, a similar explanation could account for the absence of any cases of triploidy among the larvae cold-treated shortly after insemination by the Fankhauser-Griffiths method. There is, of course, no direct evidence for such a theory. Although exact records of mortality were not kept, it is perhaps significant that many of the embryos developing from *T. torosus* eggs treated by this technique died early in development. Indeed, a considerable number of experiments on single egg clutches subjected to cold shock shortly after insemination are not included in this report because the embryos died before a cytological study could be made. The possibility remains that a larger number of cases than that reported here (26) might reveal instances of triploidy after appropriate temperature shock treatment.

Fankhauser (1945) has pointed out that eggs from some *Triturus viridescens* and axolotl females are refractory to cold treatment, and produce predominantly diploid embryos. A similar difference in resistance to temperature shock may exist between species of Triturus. In any event, it is quite surprising that in a total of more than 2200 *T. torosus* larvae (normal, shipped and cold-treated) examined by Dr. Costello and the author, no completely polyploid individuals have yet been found.

B. Mosaicism

As by-products of parthenogenesis, cold, colchicine and heat experiments, and as the direct result of temperature shock later in development than that designed to

produce triploidy, the production of complex mosaics has been reported in the literature over a period of years. These mosaics often possess radical mitotic abnormalities as an accompanying feature.

Parmenter (1933, 1940) found haploid diploid, diploid/triploid and diploid/triploid/tetraploid individuals in parthenogenetic *Rana fusca* and *R. pipiens* larvae.

In a group of 100 refrigerated *Triturus viridescens* eggs, Griffiths (1941) found one haploid/triploid and five haploid/diploid larvae. Costello (1942) reported two haploid/diploid mosaics in a total of 100 larvae raised from cold-treated *Triturus similans* (granulosus) eggs. Barber and Callan (1943) studied tail-tips of cold- and colchicine-treated *Triton vulgaris* larvae, and described tetraploid mitoses resulting from these treatments. Böök (1943) chilled *Triton taeniatus* eggs later than half an hour after fertilization but before the first cleavage. The duration of treatment is not clearly specified, but appears to have been for periods of 6 to 15 hours. He reports a high mortality in the experimental eggs, only six of sixty-two cases surviving long enough for cytological studies to be made. All six survivors were complex mosaics of various types. In another series of experiments Böök (1945) treated *Triton taeniatus* and *Triton cristatus* eggs 4 to 15 hours at 2° C., more than thirty minutes after insemination. Thirteen embryos of the sixty-four treated survived to the neurula stage, at which time they were examined cytologically. Six of these thirteen cases were complex mosaics. A great variation in nuclear size and a number of mitotic abnormalities were also noted.

Briggs (1947) applied the heat-treatment technique of Fankhauser and Watson (1942) to anuran material. He treated 347 *Rana pipiens* eggs at various intervals after insemination, at temperatures of 35° and 37.5° C. for 4 to 7.5 minutes. Briggs found 32 "mixed" abnormal neurulae (9.2 per cent), which appear to have been haploid/diploid or haploid/triploid mosaics.

Fischberg (1947) subjected eggs of *Triton alpestris* to cold shock within 30 minutes after insemination. Among 553 larvae studied cytologically by the tail-tip method, he observed 27 haploid/diploid, 20 haploid/triploid, 5 diploid/triploid, and 1 diploid/triploid/tetraploid mosaic individuals. Eight larvae were non-mosaic aneuploids, and the remainder were euploid (haploid, diploid, triploid and tetraploid).

In general, the results of a study of the cold-treated *Triturus torosus* embryos are quite comparable to those discussed above. The incidence of chromosomal mosaicism was significantly higher than in the control larvae, although it was not as high as that (43 per cent for a sample of 126 tips) reported for *T. torosus* embryos subjected to shipment (Costello and Henley, 1947, 1948). This is understandable in view of the fact that embryos shipped for a considerable distance in ice and water are subjected to low temperatures for several days. Most of the experimental treatments reported here were not so prolonged. A few tests were made for comparable periods, but the embryos died before they could be tail-clipped. The "D" group of larvae cold-treated in 1942 shows a remarkably high frequency of mosaicism (5 larvae of 13 treated for 18 hours, beginning 80 minutes after insemination). This is a considerably longer period of cold-treatment than the majority of the experiments reported here. The high degree of effectiveness of the cold shock in producing mosaicism in this group of

eggs may be due to the fact that the treatment was operative during an especially susceptible physiological state of the zygote.

The mosaics found in the study of cold-treated embryos were much more radical than those occurring in the "natural population" studied as a control series (Costello and Henley, 1949). This is in contrast to the qualitative similarity of mitotic abnormalities in the control and experimental tips. Two of the three mosaic tail-tips observed among the controls were apparently diploid/hyperdiploid, and the third was diploid/tetraploid. One of the diploid/hyperdiploid tips was from an animal collected and raised under constant temperature conditions. The remaining two were from larvae which had been collected in very cold water. None of these three cases was as markedly atypical as the majority of instances of mosaicism among the experimental larvae. It is probable that none had been exposed to as pronounced temperature shock as the experimentally cold-treated animals. This is interpreted to indicate that there may be a direct relationship between the severity and duration of the cold shock and the degree of cytological abnormality resulting in a given individual. However, Böök (1943) expresses the opinion that no such relationship holds in Triton.

The frequency of mosaicism (especially of aneuploid constituents) may be considerably higher than the data indicate since, as was noted above, many of the metaphases are too crowded for accurate chromosome counts. Evidence for this possibility is afforded by the high incidence of variations in nuclear size and nucleolar number. Böök (1945) also reports a great variation in nuclear size in cold-treated Triton larvae. Since both of these criteria are known to be indices of heteroploidy, it seems quite likely that some of the variations found represent interphase nuclei with varying chromosome numbers.

C. Mitotic abnormalities

There have been a number of descriptions of abnormalities of spindle formation in amphibian nuclei, following irradiation, cold and colchicine treatments. An early report was that of Alberti and Politzer (1924a, 1924b) who observed the effects of x-rays on embryos of *Salamandra maculosa* eight days after insemination. The corneal epithelium of these larvae was studied at varying intervals following irradiation, and a variety of anomalies of mitosis, similar to those reported in the present study, was described. Böök (1943) treated neurulae at 2° C. for 8 hours, and examined the embryos cytologically five hours after the end of the treatment. He observed a tripolar anaphase and other abnormalities of spindle formation. Barber and Callan (1943) described a number of types of mitotic abnormality observed in tail-tips from cold- and colchicine-treated *Triton taeniatus* larvae. Many of these abnormalities were very similar to the ones described in the present study (e.g., "sticking" anaphases).

In the cold-treated *T. torosus* material, the frequency of various types of mitotic abnormality is higher in the experimental tips than in the control. Furthermore, it is appreciably higher in the regenerated experimental tips than in the "first" tips. There is a possibility that cold-treatment has delayed effects on mitosis which are expressed only after the stimulus of clipping has occurred. If this is so, such delayed effects must be operative for a relatively short time, since the incidence of mitotic abnormality in third clippings is apparently not as high as in the second

clippings. This has been found to be the case in tail-tips from shipped embryos, although no data are available for experimentally cold-treated individuals. Briggs (1947) has pointed out that in heat treated *Rana pipiens* larvae, mitotic abnormalities do not show up until the blastula and gastrula stages. A comparable retarded expression of effects might be involved in these cold-treated larvae. There is also the possibility that the higher incidence in "second" tips may be explained by the fact that such preparations usually have more mitotic figures than either "first" or "third" tips, and are generally more favorable for cytological study.

The frequent occurrence of mitotic abnormalities is of considerable interest when compared with that found in tail-tips from a "natural population," although it is lower than the incidence in tail-tips from shipped larvae (unpublished data). In general, the difference is quantitative rather than qualitative, since the types of abnormality observed are comparable to those found in both normal and shipped larvae. The main exception to this statement is found in the occurrence of multipolar spindles in the experimentally cold-treated and in the shipped larvae. No such configurations were observed in control tips; they may be the result of quite radical and prolonged temperature shock, as well as of other factors.

D. The relationship between morphological and cytological characteristics

The normal appearance of many of the cold-treated larvae affords additional evidence for the idea expressed by Costello and Henley (1949) that the general morphology and pigment pattern of a given larva are not necessarily valid criteria of its chromosome condition. Presumably, there is a fairly close correlation between the size of the pigment cells and the number of chromosome sets present in euploid individuals, but this assumption cannot be taken to apply too rigidly. Thus, Fankhauser (1945) points out that such a criterion is not always reliable in the case of axolotl larvae, where the size of the pigment cells in triploids often is not clearly distinguishable from that in diploids. In view of these findings, morphological characteristics must be used with caution as a criterion of heteroploidy. The fact that *T. torosus* larvae abnormal in general appearance may be cytologically normal confirms this belief.

E. General effects of low temperature

It is interesting to speculate on the possible reasons for the special susceptibility of anaphases and telophases in *T. torosus* to the effects of cold. Costello and Henley (1949) have suggested that the lagging and lost chromosomes and chromosome fragments noted may be due to the fact that those chromosomes lacking kinetochores would be left behind in the migration of the daughter elements to opposite poles. Since no such migration has yet occurred at the metaphase, the loss of kinetochores would pass unnoticed unless the chromosomes failed to become located on the equatorial plate. It is also possible that there is a decrease in the adhesive qualities of the chromosomes or of the spindle fibres during anaphase and telophase. This would result in the higher incidence of lagging or lost elements reported at these stages.

Böök (1943, 1945) attributes the effects of cold to a partial or complete paralysis of the spindle apparatus. This might be especially conspicuous at anaphase and telophase, interfering with the normal movements of the chromosomes. He inter-

prets his observed cases of atypical metaphase configurations as being due to the loss of ability of the centromeres to orient themselves properly. Such an explanation of the facts seems rather vague, since it does not account for the actual cause or manner of loss of the capacity of the centromeres for orientation.

It is difficult to ascribe the cytological abnormalities obtained after cold treatment to any specific effect of the low temperature. Böök (1945) interprets the origin of mosaics as a result of the omission of nuclear and cell division after cold treatment, with the retention of the division rhythm of centrosomes and chromosomes. Fischberg (1947) suggests three possible modes of origin of the aneuploid constituents of mosaics: (1) loss of single chromosomes at anaphase; (2) failure of chromosome division; (3) disorderly division of chromosomes in multipolar figures. From the evidence obtained in the present study of cold-treated *T. torosus* larvae, it seems possible that any or all of these factors may be operative in the production of the observed abnormalities. Since the loss of single chromosomes at anaphase appears to occur quite frequently, it may be that this is an especially important factor, as pointed out by Costello and Henley (1949). "Physiological polyspermy" normally occurs in the fertilization of salamander eggs; thus, there is also the possibility that low temperatures affect the supernumerary sperm nuclei so that they contribute to the formation of mosaic areas instead of degenerating as is usually the case.

In general, the evidence from this study indicates that low temperatures may result in the production of chromosomal mosaics and abnormalities of mitosis, similar to those observed in tail-tips from the larvae shipped in ice and water. The relative roles of anoxia, agitation and other factors in the shipped material, however, remain obscure.

Summary

1. A study of the incidence of chromosomal mosaicism and abnormalities of mitosis has been made, utilizing tail-tip preparations made from larvae of *Triturus torosus* subjected early in development to low temperature.

2. Three hundred-ninety two "first" tail-tips and 294 regenerated "second" tail-tips have been studied cytologically from preparations made in 1948. An additional 27 preparations made in 1942 have also been studied. Similar observations were made in 1948 on tail-tips from 582 control *T. torosus* larvae.

3. The experimental larvae were exposed to low temperatures (0 to 8° C.) for varying periods of time (45 minutes to 23 hours) at stages of development from 5 minutes after insemination, through blastula, gastrula, neurula and tail-bud stages.

4. No haploid or polyploid individuals were observed, in either the control or experimental groups.

5. Of the 392 "first" tips, 153 (39 per cent) were normal diploids; 156 (40 per cent) had nuclei of variable size or variations in nucleolar number; 79 (20 per cent) had abnormal mitoses; and 4 (1 per cent) were chromosomal mosaics. Of the 294 "second" tips, 71 (24 per cent) were normal diploids; 107 (36 per cent) had nuclei of variable size or variations in nucleolar number; 102 (35 per cent) had abnormal mitoses; and 14 (5 per cent) were chromosomal mosaics. Of the 27 tips prepared in 1942 from cold-treated animals, 5 were clearly diploid, 11 had nu-

clei of variable size or variations in nucleolar number, 6 had abnormal mitoses, and 5 were chromosomal mosaics. In general, the incidence of all types of cytological abnormality was higher in the regenerated than in the "first" tips. This incidence of abnormality is significantly higher than that found in the controls.

6. The types of mosaics found included: hypodiploid/diploid/hyperdiploid; diploid/hyperdiploid; diploid/triploid; diploid/triploid/tetraploid; diploid/triploid/pentaploid; diploid/tetraploid.

7. A wide variety of abnormalities of mitosis was noted, including: "sticky" anaphases and telophases; anaphases and telophases with lagging or lost chromosomes or chromosome fragments; anaphases and telophases with bent spindles; multipolar spindles. Masses of relatively uncondensed chromatin and interphase nuclei with persistent connections between the daughter components were also present.

8. The evidence indicates that *T. torosus* larvae exposed early in development to low temperatures may suffer quite radical changes in chromosome number in some body cells, as well as less extreme abnormalities of mitosis. This is in accord with the findings of other workers for other species, and offers a clue to the possible mode of origin of the cytological abnormalities observed in *T. torosus* embryos subjected to shipment in an ice-and-water mixture.

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